THE UNIVERSITY OF ALBERTA

EPITHELIAL CHANGES CAUSED BY WARM TOBACCO SMOKE IN RABBIT ORAL MUCOSA: GROSS AND HISTOPATHOLOGIC OBSERVATIONS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "EPITHELIAL CHANGES CAUSED BY WARM TOBACCO SMOKE IN RABBIT ORAL MUCOSA: GROSS AND HISTOPATHOLOGIC OBSERVATIONS", submitted by Carl Erwin Hawrish in partial fulfilment of the requirements of the degree of Master of Science.

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ABSTRACT

Tobacco smoke is recognized to be an irritant to oral tissue. The effect of smoke temperature as a co-irritant has not been adequately evaluated.

Warm or cool tobacco smoke was applied daily to the buccal mucosa of rabbits for 27 days to determine if smoke temperature altered the response of the tissue to the smoke. The apparatus employed delivered uninterrupted streams of smoke. which reproduced normal daily smoke dosages, but not standard smoking conditions.

Tissues that received cool smoke applications initially became necrotic and ulcerated, but healed completely by the 9th day. A clinically detectable white lesion was observed at this site before the termination of the study. Tissues that received warm smoke applications became similarly necrotic and ulcerated, but failed to heal by the end of the study.

Histologic tissue sections prepared from terminal lesions in the warm smoke group revealed inflammation, epithelial hyperplasia and hyperkeratosis. Sections from the non-ulcerated portions of the terminal lesions in the warm smoke group revealed a more marked inflammation and epithelial hyperplasia as well as an abnormal "patchy" type of keratinization.

The gross and histologic changes observed support the hypothesis that the tissue response is altered by the difference in smoke temperature.

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I

OBJECTS OF THE INVESTIGATION

In the last century, workers in the health sciences have recognized that smoking is detrimental to health. Epidemiologic studies in recent decades have linked smoking and other tobacco use to various oral diseases. Although these observations lend credence to the irritating effects of tobacco smoke on oral tissue, experimental studies have added little to our knowledge of the subject.

Various factors, both related and unrelated to the tobacco smoke, have been suggested as probable irritants. Several of these factors have been studied, but the effects of smoke temperature have not been carefully evaluated.

This study attempts to evaluate the effect of tobacco smoke temperature on rabbit oral epithelium. Daily applications of tobacco smoke at varying temperatures were applied to the buccal mucosa. The gross changes at the application sites were observed and recorded daily. Biopsies of the application sites were obtained at the termination of the experiments. Tissue sections were prepared and stained with hematoxylin and eosin, periodic-acid Schiff reagent and a modification of the Mallory connective tissue stain.

II

REVIEW OF THE LITERATURE

Innumerable scientific investigations have been conducted on tobacco and its effect on various tissues. (Larson, Haag and Silvette, 1961) (Larson and Silvette, 1968). Many epidemiologic studies have linked the occurrence of benign, premalignant and malignant oral lesions with the use of tobacco in various forms. Such evidence enabled the Advisory Committee to the Surgeon General of the United States Public Health Service (USPHS) (1964) to conclude:

> "The causal relationship of the smoking of pipes to development of cancer of the lip appears to be established.
>
> Although there are suggestions of relationships between cancer of other specific sites of the oral cavity and the several forms of tobacco use, their causal implications cannot at present be stated."

The USPHS (1967) reported that current information had strengthened the association between various forms of smoking and malignancies of the buccal-pharyngeal area, but that information was inadequate for a judgement of causality. One year later as evidence mounted, the USPHS (1968) stated:

> "A review of recent retrospective studies shows a relationship of oral cancer to all forms of smoking."

Despite the strong epidemiologic evidence, a search of recent literature reveals that attempts at experimental production of lesions in the oral cavity by tobacco smoke have been relatively few and largely unsuccessful. The studies of the effects of tobacco smoke on oral and cutaneous tissue modified by local and systemic agents, as well as studies on tobacco smoke temperatures, are the framework of this review.

I The Effect of Tobacco Smoke on Oral and Cutaneous Epithelium Modified by Local Agents.

The action of the tongue and saliva in removing deposited tobacco tars from oral sites has been recognized by several investigators. (Kreshover, 1952) To evaluate the possibility that saliva decreases the susceptibility of tissue to the effect of tobacco smoke, Kreshover and Salley (1957) extirpated the major salivary glands of hamsters and subjected the palatal mucosa of these animals to 10 daily applications of cigarette smoke for 64 weeks. Smoke applications were made by a machine devised by Kreshover (1952) which ejected streams of smoke for 5 second periods every 25 seconds at a rate of 17.5 ml/second. No gross or microscopic changes were observed in the mucosa at the termination of the study. They suggested that the failure of lesions to occur was due to the appreciable oral lubrication provided by minor secreting glands in the oral cavity and an inherent tissue resistance to the effects of the smoke.

Recognizing that the hamster cheek pouch is devoid of glandular elements and removed from the influence of tongue activity and salivary flow, Kreshover and Salley (1957) conducted correlative studies on the cheek pouch mucosa of additional hamsters. No gross lesions were observed after 32 weeks of smoke applications. However, microscopic examination of the smoked cheek pouches did reveal a mild to moderate degree of inflammatory change. They concluded that the hamster cheek pouch, in contrast to the hamster palate, showed some susceptibility to the effects of cigarette smoke.

Salley (1959), in a study of the effect of cigarette smoke as a co-carcinogen, applied cigarette smoke and/or ultraviolet irradiation to the lips and ears of mice. Each animal received 10 daily smoke exposures to the lip of 5 seconds each and 5 daily smoke exposures to the ear as described by Kreshover (1952),

followed by 2 minute daily concurrent irradiation to the smoked lips and ears. He reported that approximately 8% of animals that received both smoke applications and ultraviolet light and 13% of animals that received only ultraviolet light developed a squamous cell carcinoma of the ear during the 94 week experimental period. No tissue alterations were found in the lips of either group. Salley concluded that the malignancies in this study were the result of ultraviolet irradiation and that smoke applications had no significant effect. The lack of lip lesions was attributed to protective influence of the tongue and saliva.

To evaluate the effects of heat and cigar smoke as it occurs in reverse cigar smoking, Frongia and Caruso (1962) applied these agents to the palatal mucosa of dogs for periods up to 21 weeks. The smoke was applied as a continuous stream (at undetermined flow rates) for 10 to 15 minutes daily while the heat (at undetermined temperatures) was provided by positioning the burning end of the cigar 1.5 cm. from the smoke application site. They reported that ulcerative lesions developed at the application sites at the end of the 3rd week of smoke application. Histologic sections of the lesions revealed a fairly typical non-specific necrotic and inflammatory response. They concluded that on the basis of microscopic findings, the tissue represented a response to many and varied stimuli.

In subsequent studies of smoke as a co-carcinogen, Salley (1963) applied a subtumorigenic dose of a carcinogen* to cheek pouches, palates and ears of groups of hamsters. The sites were then subjected to daily cigarette smoke applications (except Saturdays and Sundays) for 60-81 weeks. Salley reported that histologic sections from the three sites revealed frequent instances of

* 9-10-dimethyl-1, 2-benzanthracene

hyperplasia, occasional instances of dyskeratosis and only rare instances of carcinoma. Control animals that received only smoke applications revealed no histologic changes except for occasional instances of hyperplasia in the palatal mucosa. Control animals that received only an application of the carcinogen to the three sites revealed no histologic alterations except for occasional instances of hyperplasia in the cheek pouch mucosa. Salley suggested that in oral epithelium, cigarette smoke appeared to act as a cancer-promoting agent by chronic irritation.

In an earlier study, Salley (1961) had demonstrated that three applications of carcinogenic hydrocarbon were necessary for significant penetration to occur in oral epithelium. Using this evidence as a basis, Salley (1963) applied 3 applications of a carcinogen* to the cheek pouches of hamsters over a period of 6 days (1 application every 2 days) followed by daily tobacco smoke application for 92 weeks. He reported that 56% of the surviving animals developed malignancies at the application sites with an average latent period of 24 weeks. 24% of the surviving control animals that received only the carcinogen applications developed malignancies with an average latent period of 38 weeks. None of the surviving control animals that received only smoke applications developed malignancies. Salley concluded that on the basis of the shorter latent period and the higher tumor yield in animals that received carcinogen and smoke, cigarette smoke acted as a cancer-promoting agen. Wynder and Hoffmann (1967, p. 192) cite an earlier similar study by Akamatsu who also showed that tobacco smoke together with a known carcinogen causes cancer earlier than the carcinogen alone. However, the tumor yield in animals that received both smoke and carcinogen was not significantly higher than in animals that received only the carcinogen.

* 0.5% 9-10-dimethyl-1, 2 benzanthracene

Elzay (1969) investigated the effects of cigarette smoke and alcohol, alone and in combination, as promoting agents in hamster cheek pouch carcinogenesis. Each of the pouches was first painted tri-weekly with a carcinogen* for 4 weeks (12 paintings). Following this tumor induction period, the pouches were painted with alcohol** tri-weekly or subjected to daily smoke applications (except Saturdays and Sundays) from one-half a king size cigarette for 21 weeks by the apparatus described by Kreshover (1952). Some animals received both smoke applications and alcohol paintings. Elzay reported that the incidence of carcinoma was 70% in pouches that had been subjected to carcinogen and smoke; 50% in pouches subjected to carcinogen, alcohol and smoke; 40% in pouches subjected to carcinogen and alcohol; and 38% in pouches subjected to only carcinogen.

Two control groups received only smoke applications and/or alcohol paintings. Except for parabasal hyperplasia in 18% of the pouches that received both alcohol and smoke, and common hyperkeratosis in pouches that received only smoke, no significant changes were observed.

A summary of the histologic findings in this study is shown in the following table.

* 0.5% 7, 12-dimethyl benz(a)anthracene in heavy mineral oil (USP)
** 50% alcohol by volume

Group	Application	Parabasal Hyperplasia %	Dyskeratosis %	Carcinoma in situ %	Carcinoma %
I	carcinogen alcohol smoke	88	81	38	50
II	carcinogen alcohol	60	80	40	40
III	carcinogen smoke	80	100	70	70
IV	carcinogen	46	46	31	38
V	alcohol smoke	18	0	0	0
VI	smoke	0	0	0	0

Elzay's findings support previous reports that alcohol and smoke are promoting agents in hamster pouch carcinogenesis. His results illustrate that smoke is a more potent promoting agent than alcohol. Alcohol and smoke do not appear to act synergistically as a promoting agent; in fact, when alcohol was applied in combination with smoke, it appeared to decrease the cancer-promoting potential of smoke. Elzay concluded that his study supported previous reports that smoke and alcohol act as promoting agents in carcinogenesis and not as carcinogens or co-carcinogens. He stated that his evidence supports the clinical contention that a relationship exists between oral cancer in humans and the consumption of alcohol and cigarette smoke.

II The Effect of Tobacco Smoke on Oral and Cutaneous Epithelium Modified by Systemic Agents.

Kreshover (1952) studied the effect of cigarette smoke on oral and cutaneous epithelium of nutritionally deficient and/or gonadectomized mice. Each animal received 10 smoke exposures to the mucocutaneous tissue of the lip and 5 smoke exposures to the postero-superior region of the ear on alternate days for 11 weeks. Smoke applications were made by a cigarette smoking machine which delivered smoke to the selected sites for 5 second periods every 25 seconds at a rate of 17.5ml/second. Kreshover reported that no significant clinical or histologic changes occurred in the lips of experimental and control animals. He attributed these negative findings to the action of the tongue and saliva in removing deposited tars from the application site plus "the inherent protective nature" of the lip epithelium.

Smoke applications to the ears of normal diet and vitamin A-deficient animals induced a slight degree of epidermal thickening and redness. Histologic examination of this tissue revealed a moderate degree of keratinization and epidermal hyperplasia with variable degrees of inflammatory changes in the connective tissue. In contrast, the smoked ears of vitamin B-deficient animals showed marked thickening, scarring; and in many instances, excoriation. Histologic tissue sections prepared from these lesions showed correspondingly severe epithelial hyperplasia and hyperkeratinization as well as chronic inflammation. In one of a group of 22 animals, dysplastic changes were reported in the ear lesions. Gonadectomy appeared to render cutaneous tissue less susceptible to the effect of smoke. Gonadectomized animals in both normal diet and vitamin B-deficient groups were free of lip and ear lesions.

The observation that a vitamin B-deficiency state rendered mouse cutaneous tissues more susceptible to injury from cigarette smoke prompted Kreshover (1955) to conduct further studies to determine which fractions of vitamin B complex were most related to tissue susceptibility. Using the apparatus previously described (1952), he made 5 smoke applications to the ears of mice on alternate days for 11 weeks. The animals were grouped according to deficiencies in components of vitamin B complex.

Although the changes reported in the various groups were generally characteristic of the B complex-deficient group, the animals deficient in riboflavin, pyridoxine and pantothenic acid developed the severest changes. Frequent instances of loss of basal-cell polarity and increased mitotic activity as well as cellular alterations in size, shape and staining qualities were observed in these groups. The smoked ears of thiamine, biotin and niacin-deficient animals showed changes similar to those observed in adequate diet groups.

Gonadectomized, riboflavin-deficient mice developed lesions that were less severe than non-castrated mice, again suggesting that gonadectomy rendered the tissue less responsive to the effects of smoke.

Post-experimental observations for 5 months revealed very little gross or microscopic change and no evidence of intrinsic capacity for progressive breakdown or overgrowth. The healing during this period was minimal except in the normal diet group where there was a reversion to normal tissue. Kreshover noted that a definite acceleration of repair could be induced by substituting an adequate diet for a deficient one.

Kreshover concluded that riboflavin, pyridoxine and pantothenic acid were the vitamin B complex fractions primarily responsible for alterations in tissue response to the effect of smoke. His observation that animals deficient in thiamine and niacin showed only slight ear pathosis was explained in part by evidence that mice are resistant to the latter deficiency and that the former deficiency is manifested principally in the nervous system and not the skin. No explanation was offered for the slight ear pathosis in the biotin deficient group.

Wynder, Graham and Croninger (1953) reported that mice require an exposure to cigarette tar for approximately 70 weeks before carcinoma could be induced. This evidence prompted Kreshover and Salley (1957) to conduct long-term studies of the effects of cigarette smoke on oral and cutaneous epithelium of mice. Smoke applications were made daily to the lips and ears of vitamin B-deficient and/or gonadectomized animals for 76 weeks. The smoked lips revealed no significant changes at the end of the experimental period. In contrast, frequent evidence of cellular abnormalities suggesting either a precancerous change or carcinoma in situ was observed in the smoked ears of the vitamin B-deficient animals. Similar, but less severe, alterations were noted in smoked ears of animals that had been maintained on a normal diet. Gonadectomized animals, in contrast to previous findings, developed lesions as early as the 12th week. Salley (1963) reported that an extension of the above study by an additional 4 to 26 weeks failed to produce any further significant alterations.

Kreshover and Salley concluded that although the lip tissue of mice was resistant to daily smoke applications for 76 weeks, the ear tissue was susceptible and formed a precancerous type of lesion after 68 weeks of daily smoke applications. They also concluded that

the severity of this cutaneous change was considerably increased by vitamin B-deficiency and that the increased skin resistance to tobacco smoke induced by gonadectomy was not evident after 12 weeks of daily smoke applications.

Since the application of cigarette smoke to oral tissue of mice was limited by animal size to the mucocutaneous surface of the lip, Kreshover and Salley (1957) conducted a correlative study on the palates and ears of normal and vitamin B-deficient hamsters. After daily smoke applications for 64 weeks, they reported that the palatal tissue was unaltered, but that cutaneous tissue was keratotic and hyperplastic. They concluded that the palatal mucosa of normal diet and vitamin B-deficient hamsters was resistant to the effect of cigarette smoke and that nutritional deficiency apparently had no influence on the cutaneous tissue resistance.

The effect of tobacco smoke on rabbit oral epithelium modified by injections of cholesterol was investigated by Roffo (1930). The site of the injections and the rationale for injections is not clear. He administered daily 5 minute smoke applications to the animals for periods up to 43 weeks at unreported flow rates and smoke temperatures. He reported that in control animals a smooth, white elevated plaque became apparent at the application site after the 3rd week. Histologic tissue sections revealed epithelial hyperplasia and hyperkeratosis with chronic inflammatory cell infiltration in the connective tissue.

A single injection of 1 ml of cholesterol prior to smoke applications delayed lesion development until approximately the 7th week of the experiment.

Injections of 0.5 ml of cholesterol on alternate days

during the period of smoke applications resulted in lesion development during the 3rd week.

Roffo concluded that these changes were characteristic of leukoplakia. Since his rationale for cholesterol injections was not reported, the significance of his findings is not clear.

III Tobacco Smoke Temperatures.

Ingelstedt and Wallenius (1961) reported that investigations of smoke temperatures should be made under conditions that closely simulate human smoking. They emphasized that temperature determinations must be made with "very quick responding heat sensing elements" and that smoke flow velocity during each puff, since it affects smoke temperature, should be monitored during temperature determinations.

Several investigators have determined cigarette, cigarello, cigar and pipe smoke temperatures in human and artificial smoking. Their findings suggest that in some forms of normal smoking and in abnormal smoking smoke temperatures occasionally exceed oral temperature.

Quigley et al (1965) recorded oral temperatures during cigarette smoking in humans with a system of micro-miniature thermocouples held by a dental prosthesis. They reported temperatures at oral sites to be less than 35^{o} C during smoking. Ingelstedt and Wallenius (1961) assessed cigarette smoke temperatures in human smoking. The temperatures were recorded by a thermoelement 2 mm from the oral end of a cigarette holding device. They reported that as the first 40 mm of a 70 mm cigarette were smoked, smoke temperatures did not exceed 31^{o} C in either form of smoking. They did, however, observe that as the next 20 mm of the

cigarette were smoked, smoke temperatures frequently reached 68° C. They concluded that since cigarettes are rarely smoked as short as 20 mm, a noxious effect from smoke heat on oral epithelium seemed highly improbable during ordinary smoking conditions. The generalization that smoke temperatures do not exceed oral temperature unless cigarettes are smoked down to 1/3 or 1/4 of their length has been supported by McNalley (1932), Lux (1933), Mulinos and Cockrill (1938), Lathiram and Korgaonkar (1964) and by investigators cited by Wynder and Hoffman (1967, p. 132) (1967, p.547).

Studies conducted by Greene (1955) and Smyth (1959) are in disagreement with the above results. Greene determined cigarette smoke temperatures in humans by inserting a series of thermocouples at various points throughout the length of the cigarette. He reported that the temperature of the gases entering the mouth ranged from 50° C to 70° C. Smyth failed to describe the method of smoking or the method of temperature determinations, but reported a smoke temperature of approximately 50° C when the combustion zone was 25 mm from the oral end of the cigarette.

Two investigations, although not directly concerned with smoke temperatures in the oral cavity, are of interest here. Kreshover (1955) evaluated the amount of heat created at the applicator tip of smoke application apparatus by incorporating a thermocouple into the applicator tip. He reported an average temperature rise of 1.8° C above "normal" body surface temperature when smoke was ejected onto the tissue. In view of the smoke temperatures reported in normal cigarette smoking and the relatively long distance of smoke travel from the burning source of his apparatus, this temperature rise is higher than expected.

Sfougaris (1964) used an electronic thermometer inserted through the tracheostoma of laryngectomized patients to determine tracheobronchial tree temperatures before and during cigarette smoking. The cigarettes were smoked through a rubber tube connected to the tracheostoma. He reported that the average temperature at various sites in the tracheobronchial tree was higher during smoking than before smoking and that this difference was more obvious in measurements made nearer the tracheostoma. It is evident from this study that smoke temperatures exceeded tracheobronchial tree temperatures.

Cigar smoke temperatures in human smokers were studied by Lux (1933) and Greene (1955). Lux determined temperatures with a thermoelement inserted 20 mm from the oral end of a cigar whereas Greene determined them with thermocouples inserted at various points throughout the length of the cigar. Greene reported that temperatures of gases entering the mouth ranged from 40° C to 60° C. Lux reported a maximum temperature of 45° C during the burning of the third quarter of the cigar. Cigarello smoke temperatures, also studied by Lux, were similar to those for cigars. A parallel study of cigar and cigarello smoking with holders by Lux revealed that temperatures at a point 20 mm from the oral end of holders do not exceed oral temperature. The evidence presented by these two investigators suggests that smoke temperatures exceed oral temperature when cigars and cigarellos are smoked beyond the last half without a holder.

Lux (1933) and Greene (1955) studied pipe smoke temperatures in humans; Harlow (1956) studied pipe smoke temperatures in an artificial smoking device. Temperature determinations in these studies were facilitated by thermocouples. Lux reported a maximum temperature of 28° C in the smoke stream at a point 20 mm from the

oral end of the pipe. Greene reported that the temperature of the gases at the entrance to the pipe stem ranged from 30° C to 50° C. Harlow reported a range of 34° C to 41° C, but failed to identify the point in the pipe smoke stream at which the temperatures were determined.

Juxtapalatal temperatures in human pipe smokers, as recorded by a mercury thermometer attached to the pipe stem, were reported by Chapman and Redish (1960) to occasionally reach 39° C.

The inconsistency and questionable validity of investigative methods employed on studies of pipe smoke temperature render the findings inconclusive. The evidence appears to be sufficiently strong to suggest that pipe smoke temperatures may frequently exceed oral temperature.

Oral temperatures during reverse smoking were investigated by Reddy et al (1960) and Quigley et al (1965). Reddy et al reported a palatal tissue temperature of 58° C in reverse cigar smokers. Quigley et al reported maximum surface tissue temperatures at various oral sites to range from 40° C to 64° C in reverse cigarette smoking. Both investigations utilized thermocouples to detect temperatures. Although reverse smoking is not a normal manner of smoking, the associated elevated oral temperatures are significant to the present study.

III

MATERIALS AND METHODS

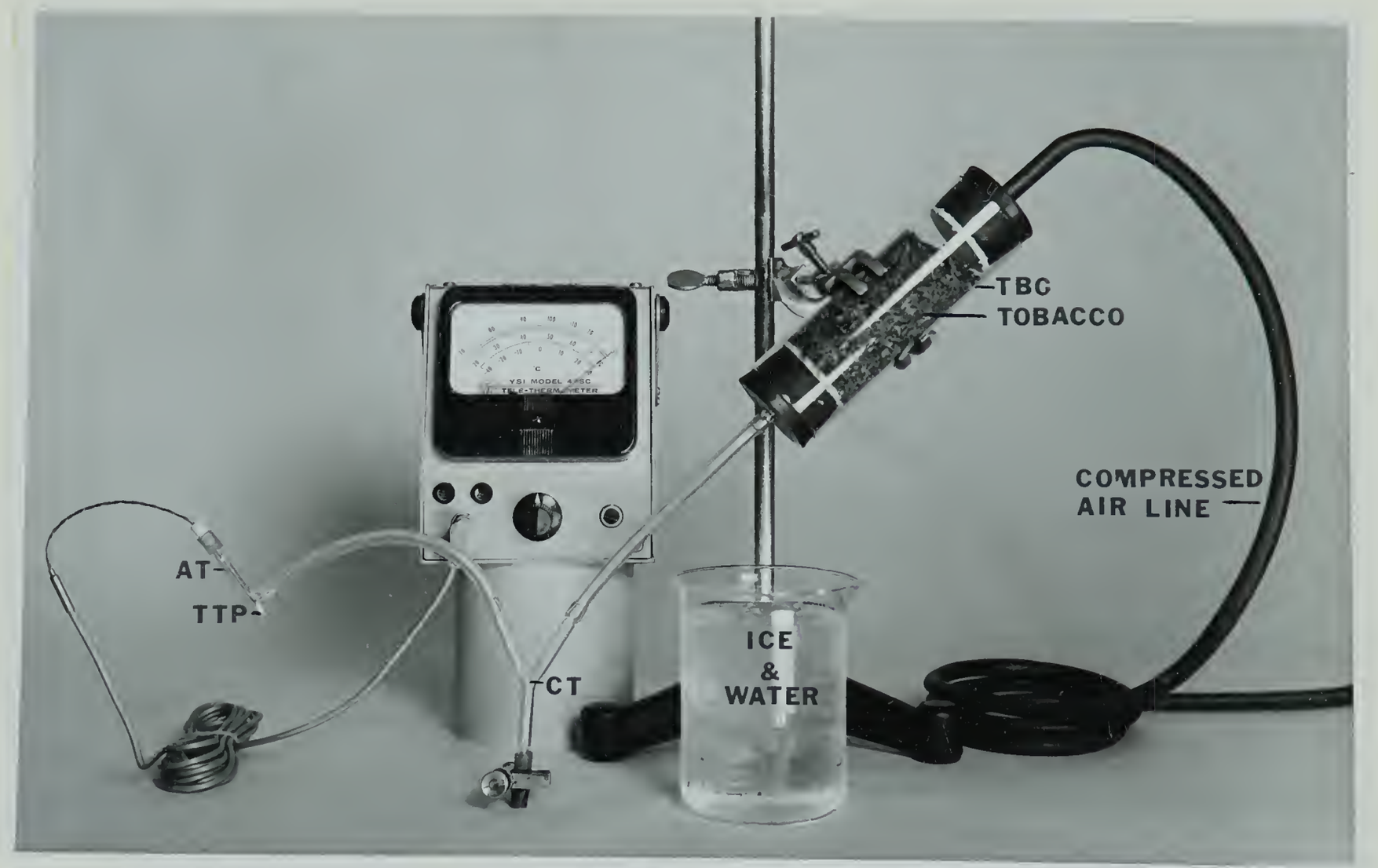

Figure 1. Smoke Application Apparatus. Tobacco burning chamber (TBC) with tobacco; compressed air line; condensate trap (CT) with pinch valve; applicator tip (AT) containing tele-thermometer probe (TTP); tele-thermometer and ice water.

MATERIALS

Forty male and female rabbits* of the New Zealand white strain were utilized for the study. At the beginning of the experiment, each animal was 10 to 12 weeks of age and weighed approximately 1.5 to 2 kg. The animals were maintained in individual wire cages at room temperature and were fed a standard balanced diet** with water ad libutum.

Five animal groups were established with equal numbers of males and females in each group. One group comprising 12 animals was subjected to cool smoke applications whereas another group of 12 was subjected to warm smoke applications. Three groups of 4 animals each received room temperature air, warm air or warm moist air applications. An additional group of 4 animals was observed during the experimental period, but received no applications.

A popular brand*** of non-aromatic pipe tobacco was used in the experiment. An apparatus was assembled which burned the tobacco and directed the smoke to the application site of the animal. The basic components of the apparatus (Figure 1) were: a tobacco burning chamber (TBC) with a compressed air supply, a glass applicator tip (AT) with a tele-thermometer probe (TTP) and interconnecting tubing with a condensate trap (CT).

The tobacco burning chamber was a Pyrex glass cylinder (47 mm inside diameter, 150 mm length) with singlehole rubber

* Purchased from A. Vandermeer, University of Alberta Farms, R.R. 3, South Edmonton, Alberta Canada.

** Master Baby Rabbit Pellets, Maple Leaf Mills Ltd., Calgary, Alberta, Canada.

*** "Sail" (Natural); purchased from local retail outlets in two-fifth pound containers.

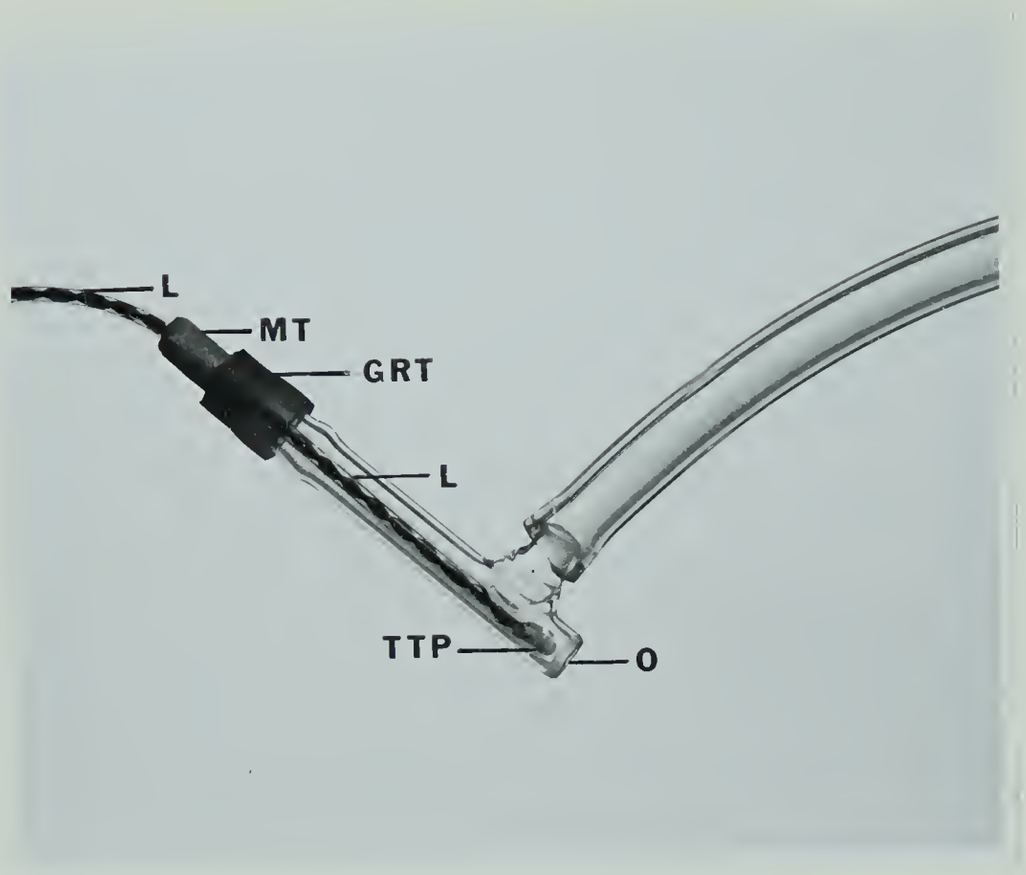

Figure 2. Applicator Tip. Lead (L); masking tape (MT); gum rubber tubing (GRT); tele-thermometer probe (TTP); near orifice (O) of applicator tip.

stoppers inserted into the ends. Asbestos discs were placed inside the chamber adjacent to the stoppers. The chamber was held by a clamp and a support stand at 35^{o} to 45^{o} from vertical.

The upper end of the tobacco burning chamber was connected to rubber tubing which carried the compressed air from the compressed air cylinder* (not illustrated). The flow rate of the compressed air was controlled by a regulator flowmeter** (not illustrated) which was connected directly to the outlet of the cylinder. The range of the flowmeter was 0.5 to 9 standard litres per minute (SLPM).

The lower end of the tobacco burning chamber was connected to the condensate trap by a 150 mm length of polyvinyl chloride tubing*** (3/16 in. inside diameter). The condensate trap was a glass "Y" shape connecting tube (5/16 in. outside diameter). Attached to the stem of this "Y" connecting tube was a short length of rubber tubing with a pinch valve distal to the stem to permit evacuation of condensate. A 150 mm length of polyvinyl chloride tubing connected the condensate trap to the applicator tip.

A glass "T" shape connecting tube (5/16 in. outside diameter) was modified to serve as the applicator tip (Figure 2). The length of the stem of the "T" shape connecting tube was reduced to 5 mm and was connected to the tubing from the condensate trap. One arm of the connecting tube was also reduced to 5 mm length and served as the applicator tip orifice. The unaltered arm of the connecting tube was attached to a short length of gum rubber tubing.

* Canada Liquid Air Ltd., Edmonton, Alberta, Canada.
** Constant Flow Air Regulator, Hoeke Incorp., Cresskill, New Jersey, U.S.A.
*** Tygon Tubing, Fisher Scientific Co., Edmonton, Alberta, Canada.

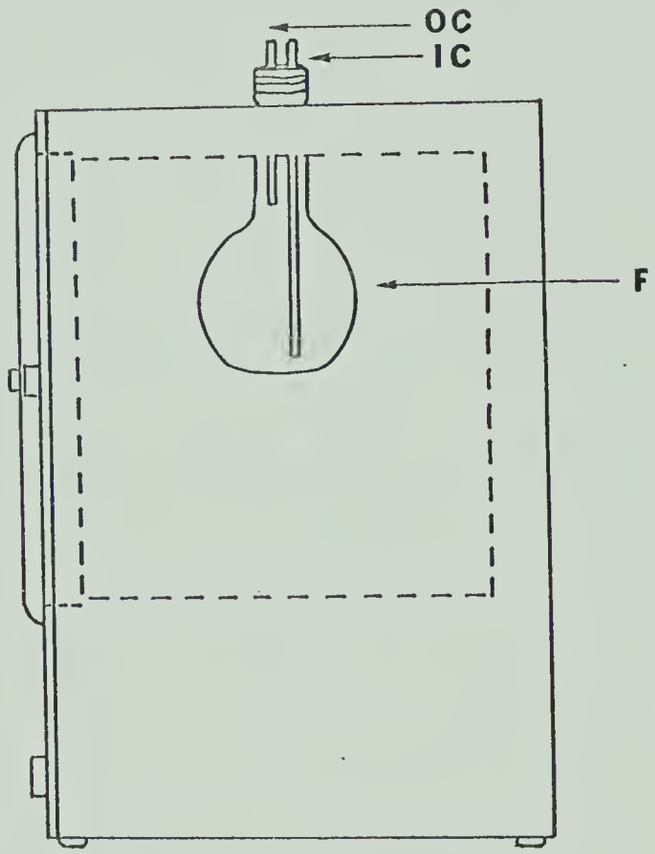

Figure 3. Warm Air and Warm Moist Air Application Apparatus. Flask (F) with inlet connector (IC) and outlet connector (OC) suspended in gravity convection oven.

The lead (L) of the telethermometer probe was wrapped with masking tape (MT) at a point which would permit it to be inserted and held snug with the telethermometer probe (TTP) 1 to 2 mm from the orifice of the applicator tip.

A small laboratory animal rectal tele-thermometer probe* was utilized. It had a maximum diameter of 3 mm, a range of -80° C to +100° C and a time constant of 3.2 seconds. The probe was coupled to a wide span, multi-range transitorized thermometer** with ranges of -40° C to +30° C, +20° C to +80° C and +70° C to +150° C. The thermometer had a reported accuracy of ±0.5° C and a readability of ±0.2° C. The accuracy of this heat sensing portion of the apparatus was verified prior to, during the course of and at the termination of the experimental period. This was accomplished by immersing the tele-thermometer probe in a water bath*** at various temperatures and comparing the temperatures recorded by the tele-thermometer with those of a previously verified mercury thermometer in the water bath.

To facilitate room temperature air applications, the applicator tip was connected to the flowmeter-regulator outlet of the cylinder air supply by a length of rubber tubing.

The air for warm air applications was warmed inside a Pyrex Florence form flask which was suspended within the working chamber of a thermostatically controlled gravity convection oven* (Figure 3). The neck of the flask protruded through the exhaust vent of the oven and was of a diameter that permitted air flow from the oven through the exhaust vent. A two-hole rubber stopper

* Fisher Scientific Co., Edmonton, Alberta, Canada
** YSI Model 42SC, University of Alberta Technical Services, Edmonton, Alberta, Canada.
*** Blue M. "Magni-Whirl", Blue M. Electric Co., Blue Island, Illinois, U.S.A.

was used to close the flask. Room temperature air from the compressed air source was led through rubber tubing into the inlet connector (IC) and warm air flowed out of the outlet connector (OC). The outlet connector was attached to a 150 mm length of polyvinyl chloride tubing leading to the applicator tip. The heat control of the oven was adjusted prior to the experiment to produce temperatures at the applicator tip within the desired range. The oven was kept operating throughout the duration of the experiment.

Warm moist air applications were not made concurrently with warm air applications. The former applications were made after the termination of warm air applications and were facilitated by the placement of 10 ml. of tap water inside the Florence flask 3 to 5 minutes prior to application. The incoming air was "moistened" as it passed through the flask. The heat control of the oven was re-adjusted prior to this portion of the study to produce temperatures at the applicator tip within the desired range.

* Precision-Thelco model 27, Precision Scientific Co., Chicago, Illinois, U.S.A.

Figure 4. Applicator Tip in Position

METHODS

The right buccal mucosa of each animal was selected as the application site. All applications were made daily (including Saturdays and Sundays) and were of a continuous nature and of five minute duration at a flow rate of 2 SLPM. Applications were continued for 27 days in all groups except the warm moist air group which received applications for 5 days.

Prior to the beginning of the study, oral temperatures of rabbits were recorded for three or more consecutive days at the time of day when smoke or air applications would be made. The calculated mean temperature was $38.8 \pm 1.2^{\circ}$ C.

Prior to application animals were placed in individual cloth restrainers. Applications were facilitated by stabilizing the head of the animal with the left hand and simultaneously retracting the lip with the thumb and exposing the right buccal mucosa. The applicator tip was held in pen-grasp fashion in the right hand at a distance of 2-4 mm from the application site (Figure 4).

1 Smoke Applications.

The apparatus was prepared for making smoke applications by the removal of the upper stopper and asbestos disc from the tobacco burning chamber and the insertion of 35 to 50 gms. of tobacco into the chamber. The tobacco was first lightly packed and then vacuum was applied to the lower stopper connection while a small flame was held to the tobacco at the upper end of the chamber. Following the ignition of the tobacco, the flame and the vacuum were withdrawn and the condensate trap, applicator tip and associated tubing were connected to the lower stopper connection. The asbestos disc and upper stopper were then inserted and the compressed air

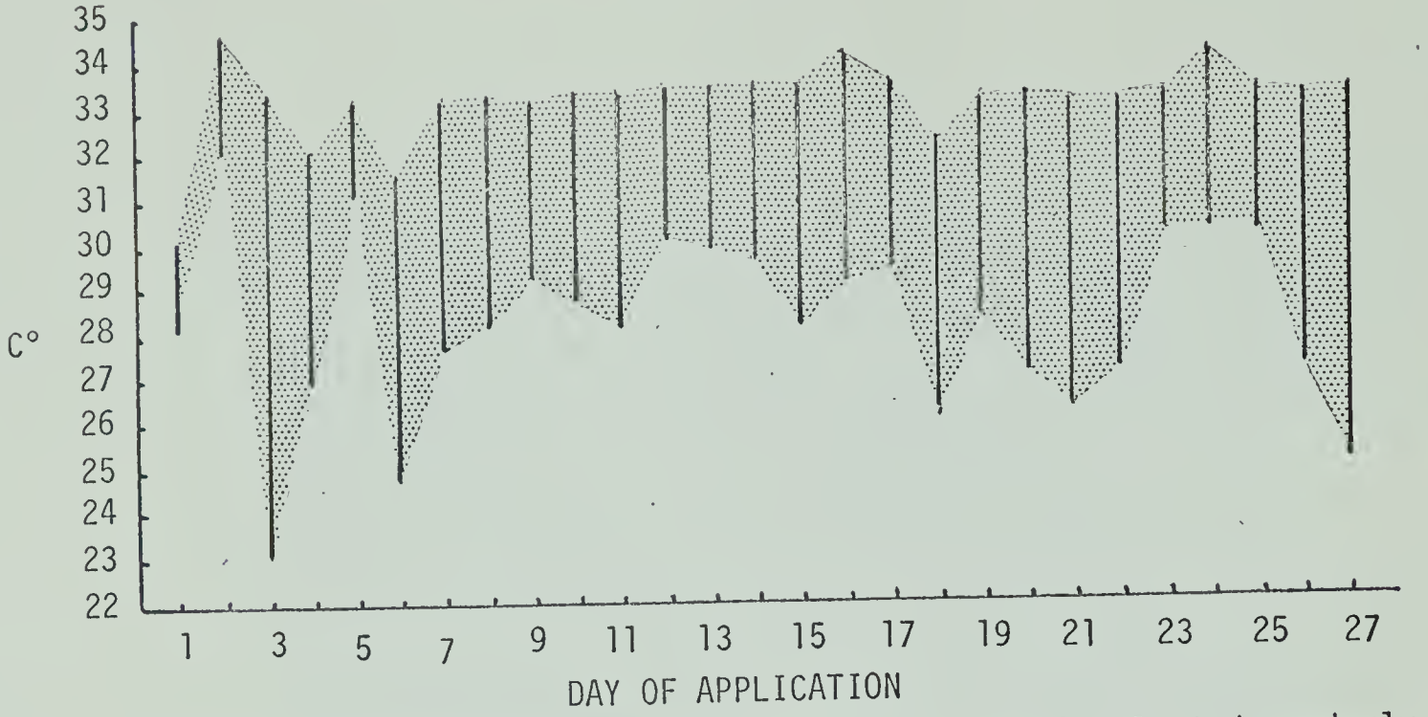

Figure 5. Daily temperature ranges for one animal selected randomly from the cool smoke application group.

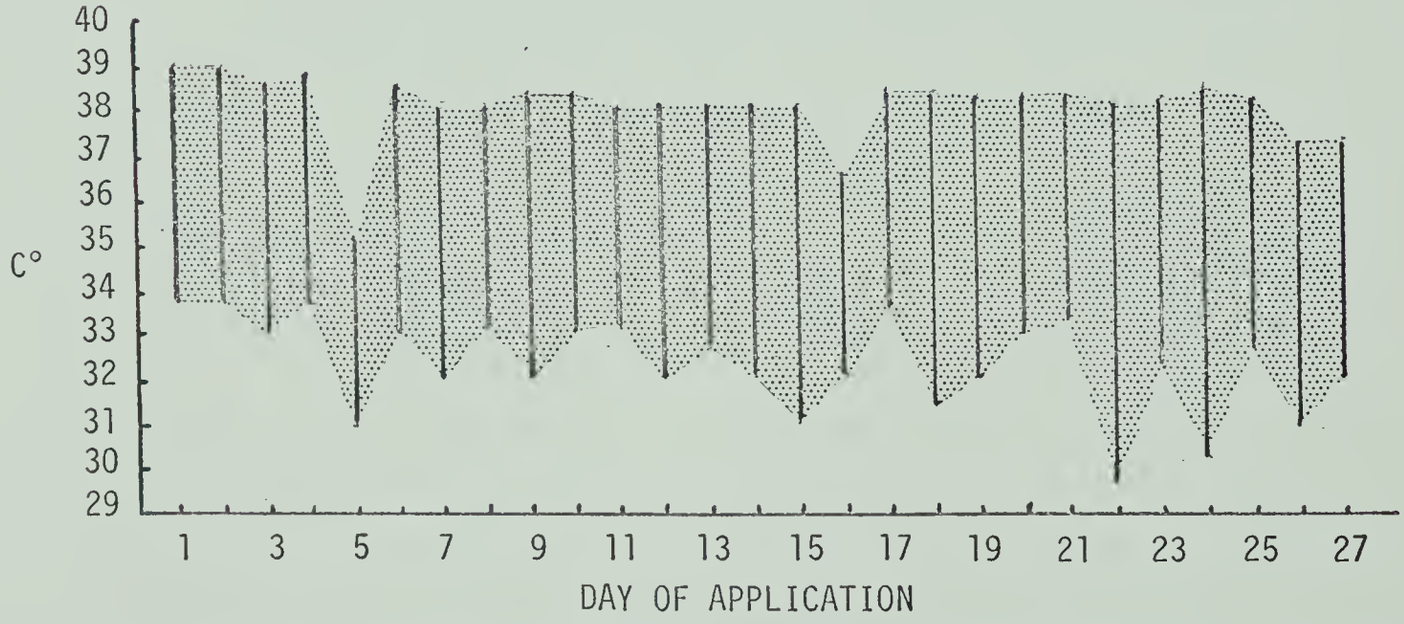

Figure 6. Daily temperature ranges for one animal selected randomly from the warm smoke application group.

supply was connected to the upper stopper connection. The extension of the combustion zone within the chamber was determined by the angulation of the chamber and followed a path 25 mm to 35 mm wide along the superior inner wall. Combustion zone temperatures were not measured.

Smoke generated by the apparatus sufficed for a series of 6 smoke applications (approximately 40 minutes operation). Because the initial combustion zone was relatively small and the chamber was cool, initial smoke temperatures at the applicator tip were in the cool smoke range. Therefore, a routine order of smoke applications was followed whereby a series of three cool smoke applications preceded a series of three warm smoke applications. The order in which the three animals received either cool or warm smoke applications was rotated daily.

Slight rotation of the tobacco burning chamber created an increased contact area between combustion zone and unburned tobacco and resulted in an increased size of combustion zone and higher smoke temperatures. Immersion of the condensate trap in ice water resulted in decreased smoke temperatures.

Cool smoke application temperatures for the entire group ranged from 18.0° C to 36.0° C. The mean minimum temperature for any animal in this group was 27.1° C whereas the mean maximum temperature for any animal was 33.1° C. The daily temperature ranges for one animal selected randomly from this group are illustrated in Figure 5.

Warm smoke application temperatures for the group ranged from 29.8° C to 40.0° C. The mean minimum temperature for any animal in this group was 32.3° C and the mean maximum temperature

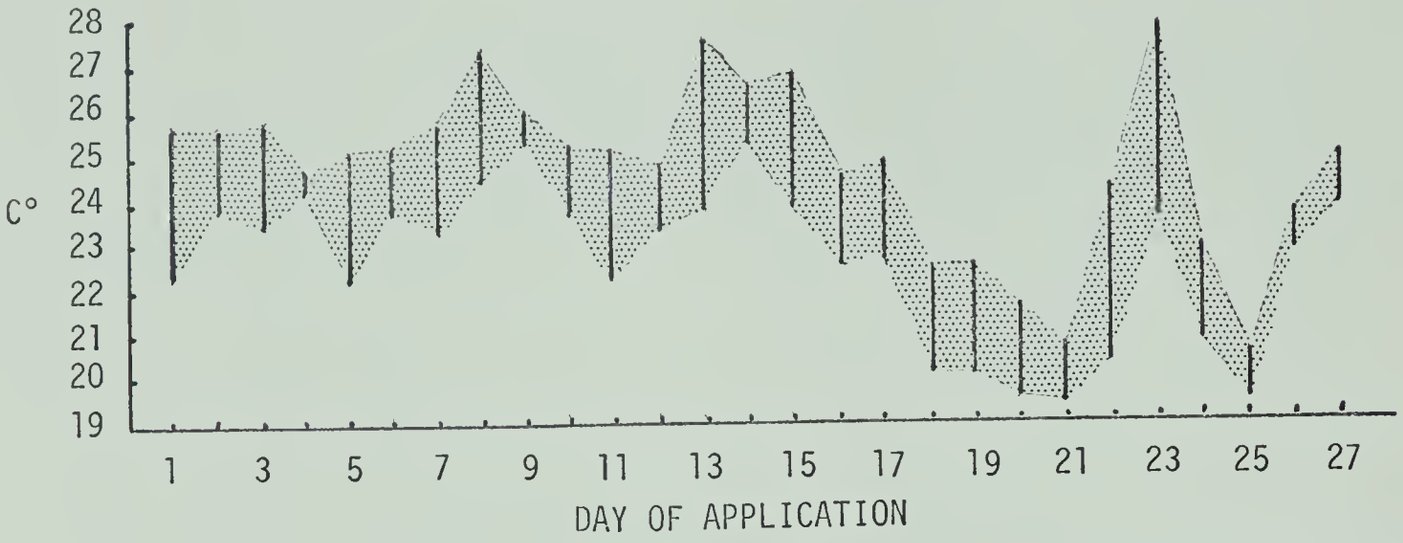

Figure 7. Daily temperature ranges for one animal selected randomly from the room air application group.

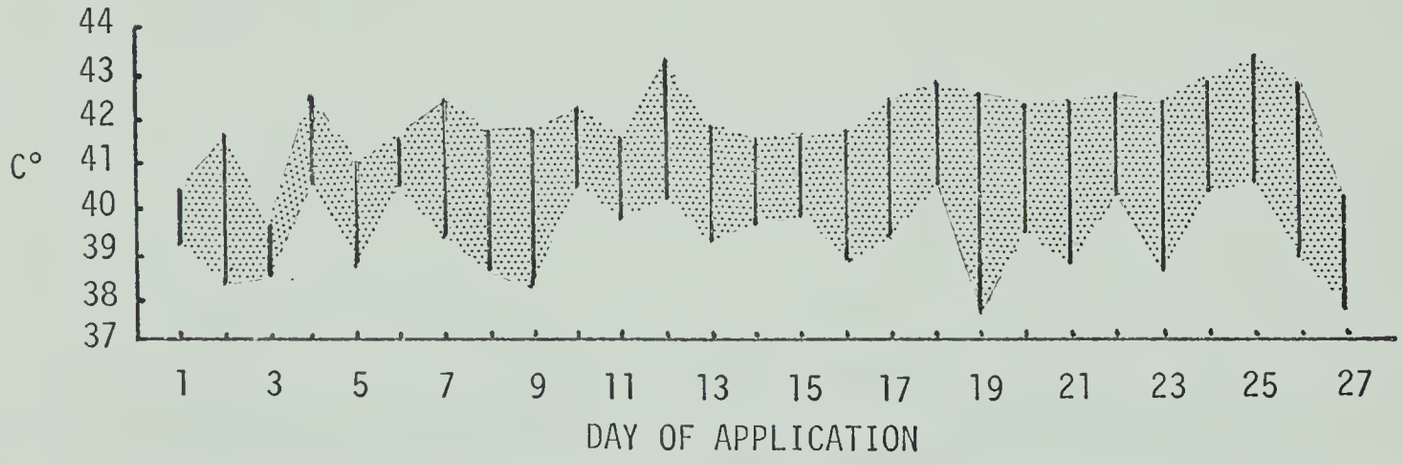

Figure 8. Daily temperature ranges for one animal selected randomly from the warm air application group.

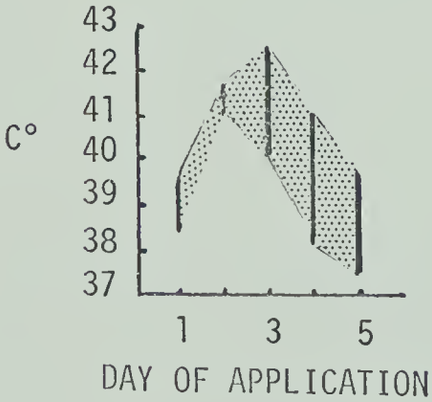

Figure 9. Daily temperature ranges for one animal selected randomly from the warm moist air application group.

for any animal in the same group was 38.7° C. The daily temperature ranges for one animal selected randomly from this group are illustrated in Figure 6.

After each series of six smoke applications, the glass components of the apparatus were cleaned and the polyvinyl tubing was replaced with new tubing.

II Air Applications.

Room air application temperatures for the entire group ranged between 18.8° C and 29.2° C. The mean minimum temperature for any animal in this group was 22.5° C and the mean maximum temperature for any animal was 27.3° C. The daily temperature ranges for one animal selected randomly from the room temperature air application group are illustrated in Figure 7.

Warm air application temperatures for the entire group ranged between 36.5° C and 43.2° C. The mean minimum temperature for any animal in this group was 38.8° C whereas the mean maximum temperature for any animal was 41.9° C. The daily temperature ranges for one animal selected randomly from the warm air application group are illustrated in Figure 8.

Warm moist air application temperatures for the group ranged between 37.0° C and 43.4° C. The mean minimum temperature for any animal in this group was 38.8° C whereas the mean maximum temperature for any animal was 41.8° C. The daily temperature ranges for one animal selected randomly from the warm moist air application group are illustrated in Figure 9.

The right buccal mucosa of all animals was observed daily and biopsied at the termination of the experiment. The specimens

were fixed in 10% formalin and embedded in paraffin. Sections were cut at approximately 6 microns and stained with hemotoxylin and eosin (H & E), periodic acid-schiff (PAS) reagent and the Ayoub-Shklar (1963) modification of the Mallory connective tissue (MMCT) stain, which stains keratin red and prekeratin-like substance orange, was used as a stain for keratin.

IV

RESULTS

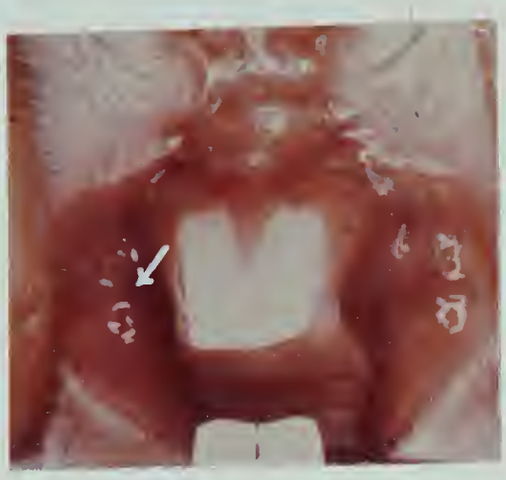

Figure 10. Application site prior to applications.

Figure 11. Lesion following 1 warm smoke application.

Figure 12. Lesion following 5 warm smoke applications.

Figure 13. Lesion following 9 warm smoke applications.

Figure 14. Lesion following 17 warm smoke applications.

Figure 15. Lesion following 27 warm smoke applications.

GROSS OBSERVATIONS

1 Warm Smoke Application Sites.

A soft, elevated, grey-white area of approximately one square centimeter in size was observed at each application site on the day following the first application (Figure 11). The surface of these areas was generally smooth, but areas of slight roughening were frequently noted. The areas were often irregular in outline and had sharp margins with moderate adjacent erythematous halos.

On the day following the second application, the grey-white areas were frequently enlarged. The elevation of the areas was more prominent and the surface was rough with evidence of some sloughing. The margins appeared to be less well-defined and erythematous halos were less prominent.

By the day following the fifth application, sloughing at the application sites was the prominent feature (Figure 12).

On the day following the ninth warm smoke application, the tissue at the application sites was completely sloughed (Figure 13). Clinical features of ulceration such as a centrally yellowish-white smooth area with sharp, slightly raised white borders were seen. The erythematous halos noted following the first and second applications were no longer evident, but a generalized moderately-white opacity of the tissue was frequently observed to surround the white borders.

Healing of the ulcerous lesion first became apparent after the ninth application and there was a notable healing after the twelfth to fifteenth applications (Figure 14). The sloughed areas had diminished in size and the borders were more raised. The

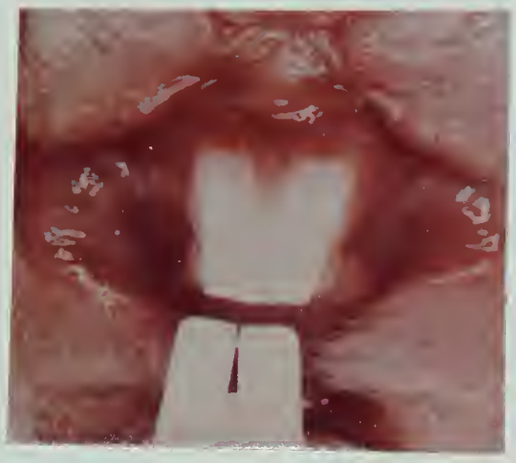

Figure 16. Lesion following 2 cool smoke applications.

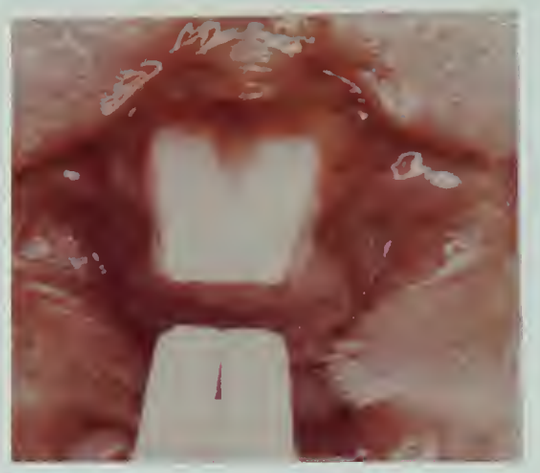

Figure 17. Lesion following 5 cool smoke applications.

Figure 18. Lesion following 9 cool smoke applications.

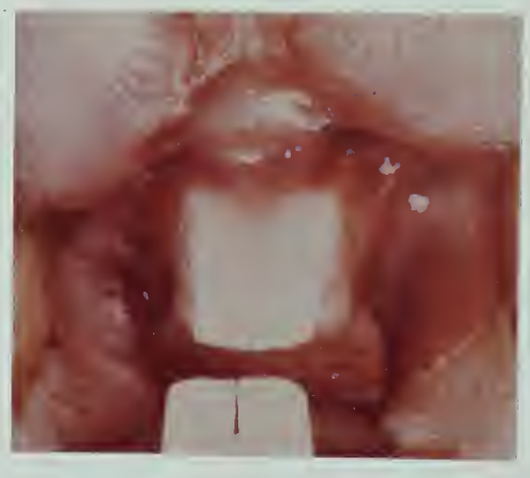

Figure 19. Lesion following 27 cool smoke applications.

white opacity of tissues surrounding the borders of the lesion became more prominent as healing progressed.

Small areas of ulceration remained at the application site at the termination of the experiment (Figure 15). The white raised borders of these lesions were surrounded by a firm whitish epithelium.

II Cool Smoke Application Sites.

On the day after the first application, application sites of animals in the cool smoke group displayed lesions similar in nature to those observed in the warm smoke group after one application. However, the areas observed in this group were frequently smaller and almost always had a smooth surface.

The involved areas, after two smoke applications, were also similar to those noted in the warm smoke group after two applications. One noteworthy difference was that areas in this group did not usually exhibit the increase in size that was observed in the warm smoke group (Figure 16).

Following the fifth cool smoke application, ulceration with sharp, very slightly raised white borders was noted, but these areas were smaller than those observed in the warm smoke group (Figure 17). Healing of these ulcerated areas became apparent after the sixth and seventh application and was completed in all animals except two by the ninth application (Figure 18). Healing of these two lesions was completed by the seventeenth application.

The tissue peripheral to the healing ulcers, as well as the ulcer site after healing was completed, displayed a firm whitish opacity with a slightly wrinkled and/or pebbly surface

(Figure 19). These epithelial alterations appeared to extend beyond the boundaries of the site of ulceration.

III Air Application Sites.

Daily examination of the application sites of room temperature air, warm air and warm moist air groups failed to reveal any gross epithelial alterations during the experimental period.

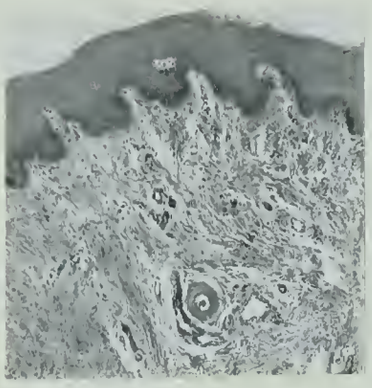

Figure 20.
Section of normal buccal mucosa. (H&E Stain).*

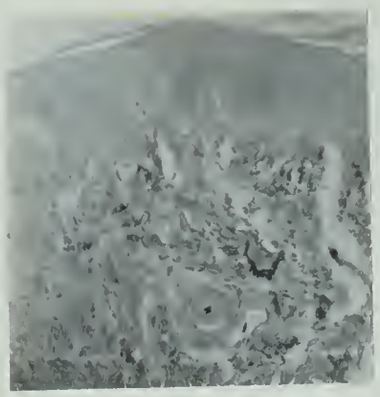

Figure 21.
Section of normal buccal mucosa. (MMCT Stain).*

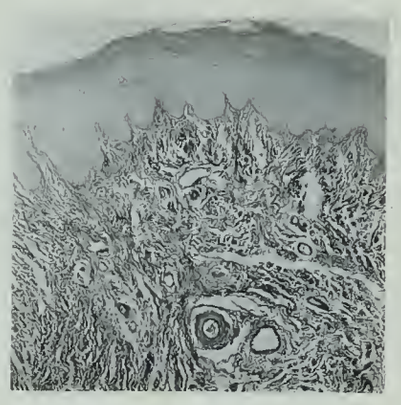

Figure 22.
Section of normal buccal mucosa. (PAS Stain).*

Figure 23.
Section of mucosa from warm smoke application site. (H&E Stain).*

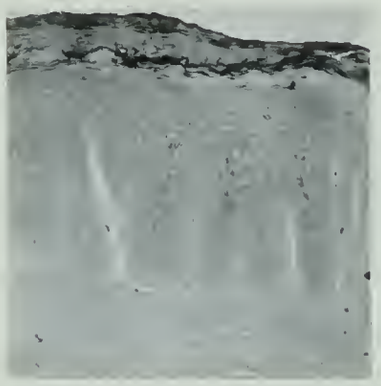

Figure 24.
Section of mucosa from warm smoke application site. (MMCT Stain).*

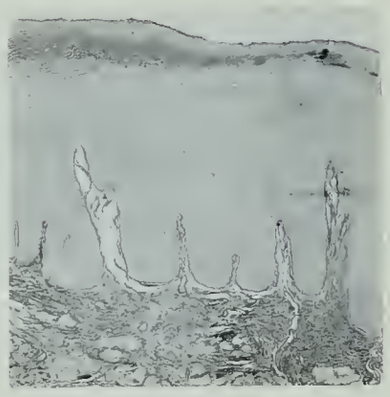

Figure 25.
Section of mucosa from warm smoke application site. (PAS Stain).*

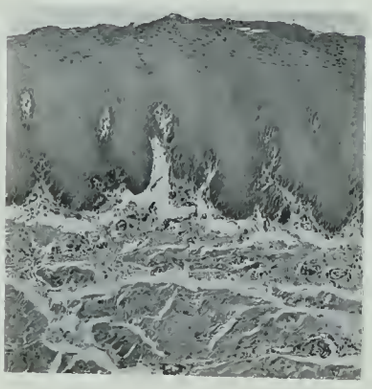

Figure 26.
Section of mucosa from cool smoke application site. (H&E Stain).*

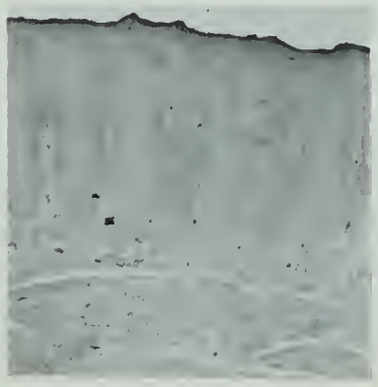

Figure 27.
Section of mucosa from cool smoke application site. (MMCT Stain).*

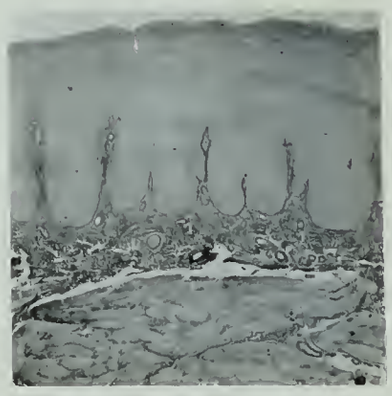

Figure 28.
Section of mucosa from cool smoke application site. (PAS Stain).*

*original magnification = 100

MICROSCOPIC OBSERVATIONS

I Control Sites.

A representative section of biopsies of the buccal mucosa of control animals stained with H & E is shown in Figure 20. The average thickness of the epithelium in these sections was 200 to 300 microns. This epithelium was composed of two or three rows of relatively undifferentiated basal cells, fifteen or twenty rows of differentiating spinous cells and three or four rows of flatter, more differentiated superficial cells in which nuclei persisted. Intracellular edema was occasionally noted in some spinous cells. Blood vessels and ducts of mucous glands were present in the connective tissue and there were practically no inflammatory cells. A similar section stained for keratin with MMCT (Figure 21) revealed that the epithelial surface was nonkeratinized and corresponded to "type 4" keratinization of the Weinmann and Meyer (1958) classification of keratinization in the human gingiva. A similar section stained with PAS (Figure 22) revealed a regular intact basement membrane between connective tissue and epithelium.

II Warm Smoke Application Sites.

A tissue section stained with H & E representative of biopsies obtained from application sites of the warm smoke group, is shown in Figure 23. The thickness of the epithelium of these lesions was significantly increased; it ranged from 400 to 600 microns. The increased thickness was due to epithelial hyperplasia, acanthosis with broad confluent epithelial ridges and frequently an increased number of more differentiated superficial cell layers. These superficial cell layers often had interspersed among them, unstained vacuolated cells with pyknotic nuclei. Intracellular edema in spinous layer cells was occasionally observed as in the control sections. Although mitotic figures in basal cells were not counted, an apparent increase

in the number of mitotic figures was observed. The lamina propria subjacent to hyperplastic epithelium was moderately inflamed with frequent plasma cells and lymphocytes, but occasional polymorphonuclear cells were also observed. Numerous small blood vessels were noted in the connective tissue, but fibrosis was not observed; in fact the connective tissue, probably due to edema, appeared less dense than control tissue. A similar section stained with MMCT stain is shown in Figure 24. Patchy areas of individual cell keratinization in the superficial, more differentiated cell layers was observed. These patchy areas of keratinization are somewhat similar to the "type 3 pattern" of keratinization in oral leukoplakia* described by Shklar (1968) but differed in that widened zones of keratin at both the base and the surface of the stratum corneum were not present. All sections of biopsies from warm smoke application sites stained with PAS (Figure 24) revealed a regular, intact basement membrane between the connective tissue and the epithelium. Areas of ulceration were not examined.

III Cool Smoke Application Sites.

A tissue section stained with H & E which had been obtained from application sites of the cool smoke group is shown in Figure 26. An increased thickness of epithelium due principally to hyperplasia and acanthosis was observed in these sections but the hyperplasia was less pronounced than in warm smoke lesions; an average thickness of 300 to 400 microns was determined. Although a slight increase in the width of the flattened superficial epithelial layers was noted, unstained vacuolated cells were not interspersed in these layers. Mitotic activity in the basal cell layers was not noticeably increased.

* A lesion in which the stratum corneum is wide and is primarily parakeratotic, but has wide zones of keratin at the base and surface of the stratum corneum as well as patchy areas of keratin throughout the stratum corneum.

A mild inflammatory infiltrate composed of plasma cells and lymphocytes was observed in the lamina propria subjacent to areas of epithelial hyperplasia. The connective tissue was not fibrotic and small blood vessels were observed less frequently in these lesions than in lesions from warm smoke application sites. A representative section of these lesions stained with MMCT is shown in Figure 27. The patchy areas of individual cell keratinization observed in warm smoke lesions were not observed in the lesions of the cool smoke application sites, but a minimal surface keratinization was frequently noted. Sections of these lesions stained with PAS revealed a normal, regular and intact basal lamina between epithelium and lamina propria. A section representing this finding is shown in Figure 28.

IV Air Application Sites.

Microscopic examination of sections from the air application sites stained by the three staining techniques utilized above revealed tissue essentially similar to that of the control sites. One significant difference was observed: biopsies from two animals of the room air application group revealed an obvious surface keratinization when stained with MMCT.

V

DISCUSSION

I Validity of the Apparatus.

In any experiments with tobacco smoke, the preparation of the smoke is of prime importance (Wynder and Hoffman, 1967, p. 94). These authors stressed that the physical form and the chemical composition of the tobacco as well as the conditions under which the tobacco is smoked affect the physiochemical properties, chemical composition and toxicity of the smoke. The nature of the apparatus used to generate smoke is, therefore, a significant factor in investigation of the effects of smoke on living tissue. Wynder and Hoffmann (1967, p. 116-120) have suggested standardized cigarette smoking conditions for studies of smoking. They advocate a puff frequency of 1-2/minute, a puff duration of 2 seconds and a puff volume of 35 ml.

The smoke application device employed in this study as well as those employed by Roffo (1930) and Frongia and Caruso (1962) ejected a continuous smoke stream and, therefore, failed to meet the standards advocated. Standard smoking conditions were adhered to in the apparatus employed by Kreshover (1952) (1955), Kreshover and Salley (1957), Salley (1959) (1963) and Elzay (1969).

Ingelstedt and Wallenius (1961) reported a peak flow velocity of approximately 2.5 liters/minute during cigarette puffs in human smokers. In contrast, Wynder and Hoffman (1967, p. 91) stated that human smokers inhale at a flow rate of 1 liter/minute.

The smoke flow rate in the present study was maintained at 2 SLPM and, therefore, compares favourably with the rate reported by Ingelstedt and Wallenius. The smoke flow rate of 17.5 ml/second in the apparatus employed by Kreshover (1952) (1955), Kreshover and Salley (1957), Salley (1959) (1963) and Elzay (1969) approximates the rate reported by Wynder and Hoffman. It is interesting to

speculate that the absence of significant positive oral findings in the studies where only smoke was applied at a liter/minute flow rate may be, at least in part, related to a lower rate of smoke flow. It can also be speculated that humans with oral lesions attributable to smoking may inhale at higher rates than those who are free of lesions.

Tobacco combustion temperatures affect the smoke both quantitatively and qualitatively. As a consequence, the toxic effect on tissue may be altered (Wynder and Hoffman, 1967, p. 127). This variable was not monitored in the present study or in any of the studies reviewed. One observation can be made concerning combustion temperatures in the various devices used and that is that the combustion of tobacco in the present study was accomplished in a closed glass chamber whereas in Roffo's study (1930) it appears to have been in a closed metal chamber. Combustion in all other studies was not accomplished in a closed combustion chamber. The significance of this difference and its effect on combustion and composition of tobacco smoke, as well as its influence on toxicity, have not been determined.

In an investigation of the effects of tobacco smoke and/or heat on tissue, the evaluation of temperature is critical. The apparatus utilized in the present study was designed to facilitate constant supervision and control of temperatures at the applicator tip. It is acknowledged that despite the presence of a rapid-responding heat sensing element in this apparatus, it was not infallible. The time constant of the probe, the readability error and the thermometer accuracy must be considered as possible sources of error. In spite of these possible errors, periodic checks and calibrations failed to reveal significant error during the course of the experiment.

In the only previous experiment on the effect of tobacco smoke and heat on tissue (Frongia and Caruso, 1962), smoke temperatures or tissue temperature at the application site do not appear to have been determined. In view of their method of positioning the burning end of a cigar 1.5 mm from the smoke application site and of the reports that combustion zone temperatures in cigars range from approximately 600° C to 900° C (Wynder and Hoffman, 1967, p. 130) one must assume that the temperature at the application site was extremely high.

Kreshover (1955) reported an average temperature rise of 1.8° C at the application site of mice during smoke application. It is significant to note that smoke temperatures at application sites in the present study did not exceed the mean rabbit oral temperature by more than that reported by Kreshover.

II The Significance of Tobacco Form, Method of Smoke Generation, and Choice of Experimental Animal.

Epidemiologic studies cited by USPHS (1968) suggest a slightly higher incidence of oral carcinoma in cigar and pipe smokers than in cigarette smokers. Various factors such as higher smoke temperature, difference in composition or concentration of smoke and difference in particular habit pattern may account for the difference in incidence. Other variables in tobacco form, such as tobacco moisture, tobacco cut, tobacco additives and tobacco packing should also be given consideration.

The selection of pipe tobacco in the present study was prompted by the epidemiologic data and by the frequent clinical observation that pipe smoking is detrimental to the integrity of oral epithelium. It is interesting to note that except for Frongia and Caruso (1962) who used cigars, and Roffo (1930) who did not

specify the type of tobacco, all other experiments have been with cigarettes. The failure to develop lesions in studies where cigarettes were used cannot, because of many other variations in methods and materials, be attributed to the difference in tobacco form. It is apparent that parallel studies on several animal species using cigarettes, cigars and pipe tobacco are indicated.

Wynder and Hoffmann (1965, p. 85) distinguish between main stream smoke (that which emerges from the oral end during puffing) and side stream smoke (that which emerges from the oral end and the burning cone during puff intermissions). The differences in the irritative properties of the two types of smoke are not known. In the present study, as well as in Roffo's (1930) study, formation of side stream smoke was not possible because of the continuous pressure upon the burning tobacco within the combustion chamber. It is assumed that the smoke in this study was not a normal main stream smoke as the lack of puff intermissions and the possible associated differences in combustion temperature would alter the composition of the smoke.

In any smoking apparatus where smoke travels from its burning source to an application site, a variable degree of condensation occurs in the interconnecting tubing. This condensation alters the composition of the smoke ejected at the application site. The problem of condensation in the apparatus employed in this study was a significant one because it was frequently necessary to chill the condensate trap (through which the smoke flowed) to cool the warm smoke or cool smoke to the desired range. None of the other studies reported a condensation along tubing but it is likely that it did occur to some degree. A detailed analysis of the composition of the condensate in this study may have revealed information relative to the irritative or carcinogenic

materials that had condensed from the smoke stream.

Uniform composition of smoke applications was not a critical point in previous smoking studies. It is acknowledged, that as a result of the rotation of the order of smoke applications for each animal, the composition of the smoke of the first 3 applications and every succeeding 3 applications was not identical. In view of the similarity of the clinical lesions that followed the first application, it is questionable that these variations were important especially at the termination of the experiment. The practice of making 3 cool smoke applications prior to 3 warm smoke applications may have been more important. The cool smoke undoubtedly differed in composition from the warm smoke because of previously mentioned condensation and because of assumed differences in combustion zone temperature. Subsequent investigations should eliminate these variables by devising an apparatus that facilitates cool and warm smoke applications of similar composition and which are independent of the heat of combustion.

The sites of smoke application in previous studies as well as the sex, strain and age of experimental animals have been varied. The present study, like the one by Roffo (1930), used the rabbit as the experimental animal. In view of Roffo's failure to report sex, strain or age of his rabbits, it cannot be ascertained that they were in all respects similar to those in this study. Even a comparison of smoke application sites in the two studies is difficult. The rabbit buccal mucosa, which has an unkeratinized epithelial surface, was used as the site of smoke application in this study. Roffo reported that he used the rabbit gingiva which is a tissue covered by orthokeratinized epithelium. It is unclear whether he in fact confined his smoke applications to the attached gingiva (which is a small zone in the rabbit), or whether smoke

was also directed against the buccal mucosa and/or palatal tissue. Furthermore, his continued references to a granular layer during the description of his histologic sections, suggest either a species difference or a microscopic interpretation error as Chen (1970) has reported absence of a granular layer in both rabbit palatal epithelium and rabbit cheek epithelium. Chen's findings are supported by evidence from the study of histologic sections obtained from control animals in the present study. If Roffo actually did apply smoke to the gingival tissue and his histologic sections include only gingival tissue, the difference between his gross and microscopic observations and those of the present study may be related to a higher resistance of keratinized epithelium to the effects of tobacco smoke.

The hypothesis that the degree of keratinization may influence the susceptibility of the tissue to the effects of smoke finds some support in the studies reviewed here. Both Salley (1963) and Elzay (1969) reported that hyperkeratosis was frequent in the hamster cheek pouch epithelium (which is unkeratinized) that was subjected to smoke applications. In contrast to this, the lip epithelium of mice (keratinized) was resistant to the effects of smoke (Kreshover, 1952; Kreshover and Salley, 1957; Salley, 1959). The hamster palatal epithelium (which is orthokeratinized) was slightly susceptible to the effects of smoke (Kreshover and Salley, 1957; Salley, 1963). The study of the palatal tissue of dogs (Frongia and Caruso 1962) is not included in this discussion because of the prolonged daily smoke applications and the assumed high heat applications.

Cutaneous smoke application sites utilized throughout various studies reviewed are, because of their glandular elements and their lack of the protective salivary action, biologically

unlike oral epithelium and have been, therefore, omitted from this discussion.

The protective action of saliva was not a variable in the present study as salivary function was unaltered. All animals were freed to their quarters upon completion of the smoke application and were, therefore, free to clean the smoke application sites. It is questionable that the susceptibility of oral tissue to the effects of tobacco smoke is in a major way related to the protective action of saliva. In view of the absence of a relationship between oral lesions attributable to smoking and xerostomia, studies of the effect of smoke on oral tissue should be made with salivary glands intact.

III The Significance of the Smoking Conditions and Smoke Dosages.

Many of the previous investigations have carefully applied smoke in a manner which simulates human smoking. While these simulations are important, they can be no more important than the total time that the smoke would be in contact with oral epithelium in an average smoker in one day. No reports have been found which state the expected average daily smoke dosage in normal smokers. Studies on cigarette consumption, by deduction, can serve this purpose. The Canada Department of National Health and Welfare (1964, p. 12) reported that of male smokers, the majority (63.8%) smoked between 10 and 20 cigarettes daily. The USPHS (1964, p. 45) cited a United States Department of Agriculture report on cigarette consumption which revealed that in 1962 each person over 15 years of age smoked approximately 11 cigarettes per day. In view of these statistics, it is apparent that in the average smoker, the daily dosage would exceed 5 minutes per day.

The present study as well as the studies by Roffo (1930) and Frongia and Caruso (1962) have reproduced these average daily

smoke dosages. Previous investigators, except for Elzay (1969) whose daily (except Saturdays and Sundays) dosage was the smoke from one-half a king size cigarette, utilized dosages of 50 seconds daily (or every other day) at oral sites. It is evident that these dosages do not reproduce the average daily smoke dosages that are expected in human smoking.

It is appreciated that a continuous smoke application of 5 minutes or more would not have the same irritative effect as several intermittent smoke applications of the same dosage. It is also appreciated that by striving to produce average daily smoke dosages, standard smoking conditions have not been maintained. The ideal experimental design should simulate human smoking conditions as well as average daily smoke dosages. Certain problems are evident in the design of such an investigation, but it is not impossible.

Studies on the particular smoking pattern of patients with oral lesions attributable to smoking are lacking. One can speculate that such studies may reveal a high incidence of smokers with relatively high daily smoke dosages, or smokers who smoke very rapidly or down to very short butt lengths. If this conjecture were to become fact, the reported elevated smoke temperatures in rapid cigarette smoking or in short butt smoking would be significant. These variables are suggested here not to de-emphasize the systemic and local influences on tissue susceptibility, but to acknowledge that other factors in smoking should be investigated.

The basis of incorporating heat as a variable in studies of the effects of smoke on oral tissue is valid (vida supra). The review literature, although inconclusive, suggests that smoke temperatures in cigar and pipe smoking may frequently exceed oral temperature. This factor may be related to the increased frequency

of oral carcinoma in cigar and pipe smokers.

IV Gross Observations.

Necrosis and ulceration were characteristic of the initial lesions that appeared at both the cool smoke and warm smoke application sites. In the studies reviewed, only Frongia and Caruso (1962) have reported a similar lesion. It is interesting to note that they reported the lesions appeared in the 3rd week of their study whereas in this study, they appeared in the first 2 days. It is somewhat surprising that the higher smoke dosages and elevated temperatures employed by these investigators did not promote a more rapid necrosis and ulceration. The only explanation that can be offered here is that the dog palate epithelium may have been, by virtue of its higher degree of keratinization, more resistant to the effects of the smoke.

Although Roffo (1930) used smoke dosages and animals similar to those in this study, he did not report an initial necrosis and ulceration at the application sites. While it is possible that such lesions did not occur due to the possible dissimilarities in smoke composition, application site or animal strain, it is also possible that they did occur, but that he reported only on the appearance of terminal lesions.

The failure to observe initial necrosis and ulceration in other studies may have been related to the lower smoke dosages employed and in case of the mouse lip and the hamster palate (Kreshover 1952, Kreshover and Salley 1957, Salley 1959, 1963), to the resistance imparted by higher degrees of keratinization.

The possibility of some degree of necrosis and ulceration preceding an oral lesion attributable to smoking in humans cannot

be readily disproved. Bloodgood (1914) reported on a lesion which he termed a "smokers' burn" in which there was distinct ulceration. An authority quoted in a communication from the New York Institute of Clinical Oral Pathology (1942) classified this shallow ulcer of "smokers' burn" with other precancerous lesions resulting from smoking. References related to this subject are distinctly lacking in the recent literature.

At least one case examined by this writer supports the contention that an oral ulceration does occur in some smokers. The patient, a young adult male, presented with ulceration of the buccal mucosa and gingiva. The lesions occurred 1 to 2 days after he began smoking a pipe. Observation of his pipe smoking pattern revealed that he held the pipe in an abnormal manner and directed the smoke stream onto the ulcerated area. The areas was healed 10 days after he stopped smoking the pipe.

The process of tissue repair at the application sites in the present study was an important clinical observation. In contrast to the healing at cool smoke sites, healing at the warm smoke sites was incomplete at the termination of the study. A basic similarity between the incomplete healing of the warm smoke site of this study and the site employed by Frongia and Caruso's (1962) study is evident. Further comparison of the mechanisms involved in this similarity is rendered invalid because of the absence of control animals in their study as well as the elevated tissue temperatures employed.

While the present study suggests that the incomplete healing of lesions in the warm smoke group is directly related to higher smoke temperatures, such a conclusion is not possible when it is recalled that the composition of warm smoke and cool smoke may not have been similar.

The terminal lesions in the cool smoke group of this study were characteristically whitish and firm with a slightly irregular surface. These appear, except for the surface irregularity, to be similar to the smooth white elevated plaques described by Roffo (1930).

V Microscopic Observations.

It is to be appreciated that the discussion of microscopic observations in this study has 2 limitations. The first limitation is that all histologic observations were made on only the terminal lesions. The second limitation is that all observations were made on tissue with an intact epithelial surface.

Inflammatory cells were consistently observed in sections of tissue that received smoke applications. The warm smoke tissue, however, differed from the cool smoke tissue in that it displayed a more pronounced degree of inflammation and occasional presence of polymorphonuclear cells. This observation is not surprising in view of the frank ulcerations which were present in the tissues that were subjected to warm smoke applications. Both of these tissues showed considerably more inflammatory changes than control tissues. These observations suggest that the differences in inflammation may be due to the differences between warm smoke and cool smoke. Such a conclusion is not possible because of the possible dissimilarity between the two types of smoke, but it is possible to conclude that the presence of inflammation is due to the irritative effects of smoke.

Inflammatory changes reported by Roffo (1930), Kreshover and Salley (1957) and Elzay (1969), though not described in detail, support the conclusion that inflammation occurs in oral epithelium during smoke applications. Differences in inflammatory response between the latter two studies and the one presented here may be

related to daily smoke dosages, smoke composition and tissue resistance. The observations on inflammatory changes by Frongia and Caruso (1962) are of questionable significance here due to the elevated tissue temperatures, high daily smoke dosages and lack of control animals.

The presence of an intact basement membrane in both experimental and control tissue in this study is not surprising. The disruption of basement membrane integrity is one of several changes that occur in long-standing highly malignant carcinomas. It is doubtful that such a change could be induced by 27 daily applications of tobacco smoke.

The observation of more mitotic activity in warm smoke tissue in this study, although it was not assessed in detail, is an observation that is compatible with changes that occur during repair at the periphery of an ulcer.

Acanthosis and epithelial hyperplasia were observed in this study in both warm smoke and cool smoke tissue. As evidenced by the increased thickness of epithelium, these changes were more pronounced in the warm smoke tissue. This difference may be attributable to the higher degree of inflammation and edema observed in the tissue subjected to warm smoke.

Surface changes in epithelium are an interesting part of the microscopic observations in this study. Tissue that had been subjected to cool smoke applications demonstrated a mild hyperkeratosis. This change is in contrast to the absence of epithelial surface change in all control animals (except 2 animals that received room temperature air applications). This observation suggests that smoke applications, at temperatures below body

temperature, can promote keratinization in tissues that are unkeratinized. This contention is supported by Elzay's (1969) report that hyperkeratosis was common in hamster cheek pouches that had been subjected to his smoke applications for 21 weeks and by Roffo's (1930) report of hyperkeratosis in rabbit oral epithelium subjected to his smoke applications. The problem of comparing Roffo's histologic findings to those of the present study has been previously elaborated upon.

Surface changes in epithelium of sections from warm smoke application sites were very different from the cool smoke sites in that a peculiar patchy type of keratinization was observed. While these changes are somewhat similar to the "type 3 pattern" of keratinization in oral leukoplakia reported by Shklar (1968), it cannot be concluded that they are in fact the same. Furthermore, while this study may suggest that this abnormal pattern of keratinization was related to the effects of warm smoke, it is difficult to disprove that it was related to the inflammation in the adjacent tissue.

The reports by Frongia and Caruso (1962) that a slight hyperkeratosis occurred in non-ulcerated areas of the dog palate subjected to smoke applications neither supports nor disproves the contention that an abnormal keratinization pattern may occur in tissue subjected to warm smoke.

VI

SUMMARY AND CONCLUSIONS

Warm and cool tobacco smoke applied by the apparatus and method described here, produced clinical and histologic alterations in the buccal mucosal tissue of rabbits. The buccal mucosa subjected to cool smoke initially became necrotic and sloughed to form an ulcer which healed rapidly. At the termination of the study, this tissue was characterized microscopically by mild degrees of inflammation, epithelial hyperplasia and hyperkeratosis. The buccal mucosa subjected to warm smoke initially formed a similar ulceration, but the ulceration failed to heal. The tissue adjacent to the remaining ulcers displayed more advanced degrees of inflammation and epithelial hyperplasia as well as an abnormal patchy type of keratinization.

Since the mucosa of control animals subjected to applications of room temperature air, warm air and warm moist air failed to reveal similar changes, the changes in tissues subjected to smoke are attributable to the effects of smoke and are perhaps modified by the temperature of the smoke.

The differences in gross lesions in the 2 smoked tissues, while they appear to be related to the effects of the smoke temperature difference, may be related to a possible dissimilarity in smoke composition. The differences between the histologic observations on the smoked tissues may also be related to the smoke temperature difference and the possible dissimilarity in smoke composition, but they may in addition be related to presence or absence of ulceration in the adjacent tissue.

From this study, it can be concluded that the tissue alterations produced by smoke at temperatures that slightly exceeded "normal" oral temperatures, are more severe than those produced by smoke at lower temperatures.

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